Title: Prolonged treadmill running in normobaric hypoxia causes gastrointestinal barrier permeability and elevates circulating levels of pro- and anti-inflammatory cytokines

Author List: Garrett W. Hill[1], Trevor L. Gillum[2], Ben J. Lee[3], Phebe A. Romano[1], Zach J. Schall[1], Ally M. Hamilton[1], Matthew R. Kuennen[1]

Author Affiliations:
[1]Department of Exercise Science, High Point University, High Point, NC 27268
[2]Department of Kinesiology, California Baptist University, Riverside, CA 92504
[3]Occupational Performance Research Group, University of Chichester, Chichester, UK PO19 6PE

Contact Information for Corresponding Author:
Matthew R. Kuennen, PhD
Department of Exercise Science
High Point University
One University Parkway
High Point, NC 27268
Phone: (336) 841-9541
Fax: (336) 888-6394
Email: mkuennen@highpoint.edu

Contact Information for Other Authors:
Name: Garrett W. Hill
Email: ghill1@highpoint.edu

Name: Trevor L. Gillum
Email: tgillum@calbaptist.edu

Name: Ben J. Lee
Email: B.Lee@chi.ac.uk

Name: Phebe A. Romano
Email: promano@highpoint.edu

Name: Zach J. Schall
Email: zschall@highpoint.edu

Name: Ally M. Hamilton
Email: ahamilto@highpoint.edu

ABSTRACT

PURPOSE: This study examined the impact of treadmill running in normobaric hypoxia on gastrointestinal barrier permeability and the systemic inflammatory response. **METHODS**: Ten recreationally-active participants completed two 1h bouts of matched-workload treadmill exercise(65% normoxic VO_{2max}) in counterbalanced order. One bout was performed in normoxia(NORM: F_IO_2=20.9%) and the other in normobaric hypoxia(HYP: F_IO_2=13.5%). Minute ventilation(V_E), respiratory rate(R_R), tidal volume(V_T), oxygen consumption(VO_2), carbon dioxide production(VCO_2), respiratory quotient(RQ), and heart rate(HR) were measured with a metabolic cart. Peripheral oxygen saturation(SpO_2) was measured with pulse oximetry. Absolute tissue saturation(StO_2) was measured with near-infrared spectroscopy. Fatty acid-binding protein(I-FABP) and circulating cytokine concentrations(IL-1Ra, IL-6, IL-10, TNFα) were assayed from plasma samples collected Pre, Post, 1h-Post, and 4h-Post exercise. Data were analyzed with 2-Way(Condition*Time) RM ANOVAs and Newman-Keuls *post hocs* were run where appropriate($p<0.05$). **RESULTS**: As compared to NORM, 1h of treadmill exercise in HYP caused greater($p<0.05$) changes in V_E(+30%), R_R(+16%), V_T(+10%), VCO_2(+18%), RQ(+16%), HR(+4%), SpO_2(-16%) and StO_2(-10%). Gut barrier permeability and circulating cytokine concentrations were also greater($p<0.05$) following HYP exercise, where I-FABP was shown increased at Post(+68%) and IL-1Ra at 1h-Post(+266%). I-FABP and IL-1Ra did not change following NORM exercise. IL-6 and IL-10 increased with exercise in both study conditions but were increased more($p<0.05$) following HYP exercise at Post(+705% and +127%; respectively) and 1h-Post(+400% and +128%; respectively).

KEY FINDINGS:

- Normobaric hypoxia caused significant desaturation and increased most cardiopulmonary responses by 10-30%.
- Significant gut barrier permeability and increased pro- and anti-inflammatory cytokine concentrations could promote an “open window” in the hours following HYP exercise.

Key Words: altitude training, endurance exercise, cardiopulmonary response, gastrointestinal barrier, inflammation, exercise immunology

INTRODUCTION

Hepatosplanchinc perfusion is reduced by 56% (Nielsen et al. 2002) to 79% (Rehrer et al. 2001) during submaximal exercise (60–75% VO_{2max}) in normoxic conditions (F_IO_2 = 21%). This hepatosplanchinc shunting is necessary to meet the increased perfusion demand of skeletal muscle during exercise. Interestingly, when the same exercise is performed in normobaric hypoxia (F_IO_2 = 11–14%) skeletal muscle perfusion requirements rise an additional 12 to 30% (Joyner and Casey 2014; Rowell et al. 1986), suggesting that hepatosplanchinc shunting may be even more severe. This is a concern because our group (Falgiano et al. 2018; Kuennen et al. 2011; McKenna et al. 2017; Szymanski et al. 2018) and other researchers (Pires et al. 2017) have shown hepatosplanchinc shunting damages the gastrointestinal (GI) barrier, allowing lipopolysaccharide (LPS)-containing gram negative bacteria to enter the blood circulation. These findings have primarily been shown during exertional heat stress, where gut barrier permeability and LPS in circulation are recognized to contribute to the pathophysiology of exertional heat stroke (EHS) (Bouchama et al. 1993; Graber et al. 1971; Lim and Mackinnon 2006), which is characterized by elevated circulating concentrations of tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6) (Bouchama et al. 1993) and lower concentrations of interleukin 10 (IL-10) (Welc et al. 2013). Of note, individuals that develop acute mountain sickness (AMS), which is a common illness during travel to high altitude (Burns et al. 2018), also exhibit elevated circulating concentrations of inflammatory cytokines (TNF-α, IL-1β, and IL-6) (Wang et al. 2018) and lower levels of anti-inflammatory cytokines (IL-10 and IL-1Ra) (Liu et al. 2017). In contrast, higher levels of anti-inflammatory cytokines have been shown to afford protection against AMS (Julian et al. 2011).

Given these facts, our group suspects that gut barrier permeability may influence host inflammatory/anti-inflammatory immune responses during normobaric hypoxia exercise and the subsequent recovery period. However, despite the strong biologic plausibility for our speculation on this mechanism, the available literature does not provide conclusive evidence to support or refute this possibility. To our knowledge, there is only one published study that has examined the plasma intestinal fatty acid binding protein (I-FABP) response to normobaric hypoxia exercise (Lee and Thake 2017). For reference, I-FABP provides a sensitive index of GI barrier permeability in response to exercise stress (Morrison et al. 2014) and that published study was performed by a member of our research group (Lee and Thake 2017). Although plasma I-FABP concentrations were shown to rise following normobaric hypoxia exercise, the primary research question in that study examined differences between 10d heat and 10d hypoxic acclimation protocols. For that reason, the study design was constrained to use an atypical exercise mode (cycling) and an exercise protocol that was of shorter duration (40min) and lower intensity (50% of normoxic VO_{2max}) than what is normally employed during normobaric hypoxia exercise training (Lee and Thake 2017).

Treadmill running is the most common exercise training mode. As compared to well-trained competitive distance runners, recreationally active individuals are more likely to utilize normobaric hypoxia to stimulate training adaptations because there is evidence that more highly trained individuals experience reductions in the absolute

training intensity that can be maintained during hypoxic exercise (Levine and Stray-Gundersen 1997; Niess et al. 2003), which leads to detraining effects. The self-selected training pace of runners has been shown to approximate the ventilatory threshold (Conconi et al. 1982), which occurs between 60-70% of VO_{2max} in recreational runners under normoxic conditions (Woltmann et al. 2015). Given this information, our group wanted to determine if recreational runners could maintain this same treadmill workload when their exercise training was performed in normobaric hypoxia. The primary purpose of this study was to examine the effect of 1 h of submaximal treadmill exercise (65% VO_{2max}) under normoxic (F_IO_2=20.9%) and hypoxic (F_IO_2 = 13.5%) conditions on systems-level physiology parameters (V_E, V_T, R_R, VO_2, VCO_2, RER, HR, SpO_2, StO_2) and blood markers of GI barrier permeability (I-FABP) and the associated immune response (IL-1Ra, IL-6, IL-10, TNFα). We hypothesized that our recreational runner cohort would experience significant reductions in oxyhemoglobin saturation and elevations in cardiopulmonary responses, GI barrier permeability, and circulating cytokine concentrations in response to normobaric hypoxia exercise.

MATERIALS AND METHODS

Participants

Ten participants (9 men and 1 woman) completed this study. Participant demographics were as follows (mean±SEM): Age 21±1 yrs; height 175±3 cm; body mass 78±5 kg; body fat 17±1%; VO_{2max} 55±3 ml/kg/min. Participants were nonsmokers, normotensive, and negative for cardiovascular, pulmonary, or metabolic disease as defined by the American College of Sports Medicine (Riebe et al. 2015). All participants were recreationally active (i.e. participated in running and other forms of exercise training for ≥5h/wk) and none of the participants had consistent exposure to altitudes above 1000m in the 6mo that preceded the study. Participants did not disclose history of altitude sickness or exercise dysfunction and were screened to ensure they were not taking any medications or supplements that might influence study outcomes.

Each participant was challenged with one hour of treadmill exercise (~65% VO_{2max}) twice; once under normoxia (NORM, F_IO_2=20.9%) and the other under normobaric hypoxia (HYP, F_IO_2=13.5%), which simulated an altitude of ~4000m. Condition order was counterbalanced and a washout period (37± 6d) was provided between study conditions. The female participant was taking a monophasic oral contraceptive, was eumenorrheic, and both of her exercise trials were conducted during the 3 weeks of stable hormone intake. All participants provided written, informed consent prior to study participation and study procedures were approved by the ethics committee of High Point University (High Point, NC, USA).

Preliminary Assessment

Participant body composition was assessed via 3-site skinfold analysis, where skinfold sites for males (chest, abdomen, and thigh) and females (triceps, suprailliac, thigh) were measured in duplicate. Body density was determined via standard regression equation and used to estimate body composition (Brozek et al. 1963). Maximal

aerobic capacity (VO_{2max}) was determined with a graded treadmill test as described previously (Falgiano et al. 2018). The treadmill workload equivalent to 65%VO_{2max} was identified and selected for use in subsequent testing. We have shown this to be a challenging yet tolerable workload when exercise is performed under a different form of environmental stress (hot/dry ambient conditions [37°C/25%RH] (Falgiano et al. 2018; Kuennen et al. 2011; Szymanski et al. 2018)).

Study Diet

Participants used a standard food log to record diet for 2d prior to each exercise trial. Diet was quantified using publicly available software (USDA National Nutrient Database for Standard Reference). Participants were given a copy of their food log from the first study condition and asked to replicate this dietary intake over the 2d prior to their second exercise trial. Dietary intake was replicated between trials because macronutrient intake has been shown to alter GI barrier permeability (Sanford and Smyth 1974). For this same reason, exercise trials were performed following an overnight fast.

Exercise Trials

Prior to each exercise trial participants were asked to refrain from exercise for 48h, alcohol and caffeine for 24h, and food consumption for 10h (i.e. overnight fast). All exercise trials were performed between 07:00 and 10:00. Participants provided urine samples at laboratory arrival for hydration assessment via urine specific gravity (REF312ATC; General Tools & Instruments, New York, NY, USA). This was done to ensure participants began exercise in a euhydrated state (USG≤1.020) because hypo-hydration degrades aerobic performance (Sawka et al. 2007). Participants next had their body mass assessed, after which they dressed in standard athletic clothing (shorts, sports bra or t-shirt, athletic socks and running shoes) and entered the environmental chamber. A patented air exchange method (CAT-12, Colorado Altitude Training, Louisville, CO, USA) was used to maintain the fraction of inspired oxygen (F_IO_2) inside the chamber at either 20.9%(NORM) or 13.5%(HYP), dependent on study condition. This environmental chamber measures 6.0m by 4.2m and requires a closed-loop airflow system to ensure F_IO_2 remains stable. Because participant heat loss mechanisms (i.e. convection, evaporation, and radiation) could potentially alter ambient conditions inside the environmental chamber, dry bulb globe temperature and relative humidity were monitored throughout exercise. A portable, calibrated metabolic cart (TrueOne 2400, ParvoMedics, Salt Lake City, UT) was used to measure the volume of oxygen consumption (VO_2), volume of carbon dioxide production (VCO_2), respiratory quotient (RQ), minute ventilation (V_E), respiratory rate (R_R), tidal volume (V_T), and heart rate (HR) of participants following 5min of standing rest and at 5, 15, 30, 45, and 60min of exercise. Total energy expenditure and oxidation rates of carbohydrate and fat during exercise were calculated from indirect calorimetry measures using standard equations (Jeukendrup and Wallis 2005). Participants also utilized the Borg scale (Borg 1982) to subjectively rate their perceived exertion (RPE) throughout exercise.

If necessary, treadmill workloads were adjusted at a participant's first exercise trial to ensure they maintained the desired exercise intensity (~65% VO_{2max}). To ensure appropriate comparisons could be made, participants replicated the treadmill workload from their first exercise trial on their second exercise trial. Participants were allowed to drink *ad libitum* in their first exercise trial, then encouraged to match that fluid ingestion rate in their second exercise trial. For reference, the average running speed on both exercise trials was 9.3±0.3km/hr and all participants completed the full 60min of exercise in both study conditions. After completing exercise participants left the environmental chamber, toweled dry, had their body mass assessed, and provided a post-exercise blood sample within 10min of exercise cessation. Further information on oxygen saturation measurements and blood collection procedures are provided below. The difference in pre- and post-exercise body mass was utilized to calculate sweat rate, which was corrected for water ingestion but not for expiratory water loss.

Oxygen Saturation Measurements

A portable continuous wave NIRS system (PortaMon, Artinis Medical Systems BV, Einsteinweg, the Netherlands) with emitting wavelengths of 750nm and 850nm was used to measure changes in tissue oxyhemoglobin, deoxyhemoglobin, and total hemoglobin. Tissue hemoglobin saturation index was calculated from these measurements to provide an index of absolute muscle tissue saturation (StO_2). The NIRS probe was positioned longitudinally on the belly of the vastus lateralis muscle, approximately 15cm above the patella. Probe site preparations included cleaning and drying of skin, after which the probe was attached to the skin with bi-adhesive tape. Per manufacturer's instructions the probe was sealed inside thin transparent plastic to protect it from sweat and keep the surface of the receiver/light sources clean. Elastic bandages were used to secure the probe to the thigh and protect it from ambient light sources. Probe placement was marked prior to the start of exercise and examined after exercise to ensure no slippage of probe had occurred. Peripheral oxygen saturation (SpO_2) was also measured throughout exercise using a standard pulse oximeter (Model 7500, Nonin Medical BV, Amsterdam, the Netherlands).

Blood Collection

Before exercise (Pre), after exercise (Post), 1h after exercise (1-Post), and 4h after exercise (4-Post) blood samples were drawn from an antecubital vein using standard venipuncture techniques. Heparinized blood was centrifuged (Cole Parmer; EW-17250-00) at 3000 RCF for 15min and plasma was aliquoted into sterile 1.7ml microeppendorf tubes that were frozen at -80°C until batch analysis of study analytes (described below). Hematocrit was determined from micro-capillary tubes that were loaded in duplicate. Hemoglobin was assayed with a kit from Sigma Aldrich (MAK115, Sigma Aldrich, St. Louis, MO, USA). A standard equation was used to calculate plasma volume changes from hematocrit and hemoglobin values (Dill and Costill 1974).

Blood Analysis

I-FABP was analyzed with an ELISA from Hycult Biotech (Plymouth Meeting, PA, USA) and IL-1Ra, IL-6, IL-10, and TNFα were analyzed with ELISAs from R&D Systems (Minneapolis, MN, USA) according to

manufacturer's instructions. Data were generated on a Synergy HT Microplate Reader from Biotek (Highland Park, Winooski, VT, USA) using Gen5 software. All samples for an individual participant were analyzed on the same plate. Information regarding the sensitivity and precision of each ELISA is provided in Table 1.

Please Insert Table 1 Here

Statistical Analysis

Statistical analyses were performed using STATISTICA for Windows (version 7.1; StatSoft Inc., Tulsa, OK, USA). Normality of data was verified using the Kolmogorov-Smirnov test. For clarity, data in text, tables, and figures are presented as mean ± SE for N=10. Differences in dietary intake, ambient conditions, exercise workload, and urine/blood measurements were determined with paired sample t-tests. Differences in V_E, R_R, V_T, VO_2, VCO_2, RQ, energy expenditure, carbohydrate and fat oxidation, HR, SpO_2, StO_2, and RPE over the 60min exercise bout were determined with two-factor RM-ANOVAs, where intervention (Normoxia or Hypoxia) and exercise time (0-60 min) served as the repeated measures factors. Differences in I-FABP, IL-1Ra, IL-6, IL-10, and TNFα were determined with two-factor ANOVAs, where intervention (Normoxia or Hypoxia) and blood sample timepoint (Pre, Post, 1-Post, 4-Post) served as the repeated measures factors. Statistical significance was set at $p \leq 0.05$. Significant main and interaction effects were further evaluated by way of Newman Keuls post hoc analysis. For all significant effects, effect sizes were calculated [as Cohen's d (*d*) for dependent t-tests or as partial eta squared (η_p^2) for RM-ANOVA] to provide the reader with an objective indication of the magnitude of the difference. For reference, values of 0.2, 0.5, and 0.8 correspond to small, medium, and large effect sizes for *d,* respectively and values of 0.01, 0.09, and 0.25 are considered to be small, medium, and large effect sizes for η_p^2 (Cohen 1992).

Power Analysis.

A power analysis was conducted using the means and standard deviations reported in the lone prior study examining changes in gastrointestinal barrier function following normobaric hypoxia exercise (Lee and Thake 2017). From this study it was determined that with an α-level of $p \leq 0.05$, ten participants would result in an 88% probability (i.e., 1 - β) of detecting differences in the I-FABP response to exercise performed under normoxic versus normobaric hypoxia conditions.

RESULTS

Equality of Study Conditions

There were no differences in dietary intake, ambient conditions, exercise workload, or hydration measures between study conditions (Table 2).

Please Insert Table 2 Here

Ventilation.

Minute Ventilation (V_E). There was a significant interaction effect for V_E [F (5,45) = 9.378, p<0.001, η_p^2 =0.510] (Fig 1A), which differed between NORM and HYP at 5, 15, 30, 45, and 60min of exercise (all p<0.001).

Respiratory Rate (R_R). There was a significant interaction effect for R_R [F (5,45) = 6.035, p<0.001, η_p^2 =0.401] (Fig 1B), which differed between NORM and HYP at 5, 30, 45, and 60min of exercise (all p<0.001).

Tidal Volume (V_T). There were significant main effects of study condition [F (1,9) = 7.519, p=0.023, η_p^2 =0.455] (Fig 1C) and exercise time [F (5,45) = 56.19, p<0.001, η_p^2 =0.862] for V_T but the interaction effect was not significant [F (5,45) = 1.913, p=0.111]. V_T differed between NORM and HYP at 5, 15, and 30min of exercise (all p<0.050). *Please Insert Fig 1 Here*

Indirect Calorimetry.

Oxygen Consumption (VO_2). There was a significant main effect of exercise time for VO_2 [F (5,45) = 194.4, p<0.001, η_p^2 =0.956] (Fig 2A), where all exercise timepoints in NORM and HYP were shown to exceed resting values (all p<0.001). Because participants maintained the same treadmill running speed in NORM and HYP (9.3±0.3km/hr), neither the main effect of study condition [F (1,9) = 2.407, p=0.155] nor the interaction effect [F (5,45) = 1.550, p=0.194] were significant. For reference, participants exercised at 63.9 ± 6.2% and 64.5 ± 6.7% of their normoxic VO_{2max} in the NORM and HYP conditions (respectively) and there was no difference in the exercise intensity between study conditions (p=0.838).

Carbon Dioxide Production (VCO_2). There was a significant interaction effect for VCO_2 [F (5,45) = 8.077, p<0.001, η_p^2 =0.473] (Fig 2B), which differed between NORM and HYP at 5, 15, 30, 45, and 60min of exercise (all p<0.001).

Respiratory Exchange Ratio (RER). There was a significant interaction effect for RER [F (5,45) = 15.86, p<0.001, η_p^2 =0.638] (Fig 2C), which differed between NORM and HYP at 5, 15, 30, 45, and 60min of exercise (all p < 0.001). Note: RQ did not increase above resting values during NORM exercise (all p>0.050) but was increased at all exercise timepoints in HYP (all p<0.001).

Energy Expenditure. There was a significant main effect of exercise time for energy expenditure [F (1,9) = 198.13, p<0.001, η_p^2 =0.957] (Fig 2D), but neither the main effect of study condition [F (1,9) = 4.95, p=0.053, η_p^2 =0.355] nor the interaction effect [F (5,45) = 1.145, p=0.351] were significant. Energy expenditure was elevated above rest at 5, 15, 30, 45, and 60min of exercise (all p < 0.001).

Carbohydrate Oxidation Rate. There was a significant interaction effect for carbohydrate oxidation rate [F (5,45) = 14.52, p<0.001, η_p^2 =0.617] (Fig 2E), which differed between NORM and HYP at 5, 15, 30, 45, and 60min of exercise (all p<0.001).

Fat Oxidation. There was a significant interaction effect for fat oxidation rate [F (5,45) = 119.63, p<0.001, η_p^2 =0.686] (Fig 2F), which differed between NORM and HYP at 5, 15, 30, 45, and 60min of exercise (all p<0.001). Note: Fat oxidation was increased above rest at all exercise timepoints in NORM (all p<0.001) but did not increase above rest during HYP exercise (all p>0.050).

Please Insert Fig 2 Here

Cardiovascular Strain.

Heart Rate (HR). There were significant main effects of study condition [F (1,9) = 15.59, p=0.003, η_p^2 =0.634] (Fig 3A) and exercise time [F (5,45) = 627.2, p<0.001, η_p^2 =0.986] for HR but the interaction effect was not significant [F (5,45) = 0.042, p=0.999]. HR differed between NORM and HYP at 0, 5, 15, and 30min of exercise (all p<0.050).

Borg Rating of Perceived Exertion (RPE). There was a significant interaction effect for the Borg RPE scale [F (5,45) = 9.352, p<0.001, η_p^2 =0.510] (Fig 3B), which differed between NORM and HYP at 5, 15, 30, 45, and 60min of exercise (all p<0.001). For reference, the qualitative aspects of the Borg RPE scale indicate our recreational runner cohort rated exercise as "Light" in NORM and "Somewhat Hard" in HYP.

Peripheral Oxygen Saturation (SpO_2). There was a significant interaction effect for SpO_2 [F (5,45) = 6.79, p<0.001, η_p^2 =0.430] (Fig 3C), which differed between NORM and HYP at 0, 5, 15, 30, 45, and 60min of exercise (all p<0.001).

Absolute Tissue Saturation (StO_2). There was a significant interaction effect for StO_2 [F (5,45) = 7.341, p<0.001, η_p^2 =0.449] (Fig 3D), which differed between NORM and HYP at 5, 15, 30, 45, and 60min of exercise (all p<0.001).

Please Insert Fig 3 Here

Summary of Systems-Level Physiological Responses. To aid in data interpretation, a summary of the absolute and relative changes in systems-level physiological parameters has been provided (Table 3).

Please Insert Table 3 Here

Gastrointestinal Barrier Permeability and Circulating Cytokines

Intestinal Fatty Acid Binding Protein (I-FABP). The interaction between study condition and exercise time was significant for I-FABP [F(3,27) = 4.036, p=0.017, η_p^2 =0.309] (*Fig 4A*). Exercise in NORM did not contribute to significant changes in I-FABP at Post, 1-Post, or 4-Post (all p>0.050). Whereas, exercise in HYP caused a 68% increase in I-FABP at Post (p<0.001) before returning to baseline values at 1-Post, then falling below baseline at 4-Post (-44%, p=0.008).

Interleukin 1 Receptor Antagonist (IL-1Ra). The interaction between study condition and exercise time was significant for IL-1Ra [$F(3,27) = 3.520$, $p=0.028$, $\eta_p^2 = 0.281$] (*Fig 4B*). Exercise in NORM did not contribute to significant changes in IL-1Ra at Post, 1-Post, or 4-Post (all p>0.050). Whereas, exercise in HYP caused a 266% increase in IL-1Ra at 1-Post ($p=0.001$) before returning to baseline values at 4-Post ($p=0.798$). Post hoc analyses further indicated that the increase in IL-1Ra at 1-Post in HYP exceeded IL-1Ra levels at 1-Post in NORM ($p=0.001$).

Interleukin 6 (IL-6). The interaction between study condition and exercise time was significant for IL-6 [$F(3,27) = 5.271$, $p=0.005$, $\eta_p^2 = 0.369$] (*Fig 4C*). Exercise in NORM increased IL-6 by 472.9% at Post (p<0.001), by 274% at 1-post (p<0.001), and by 133% at 4-Post (p=0.020). In HYP IL-6 increased by 705% at Post (p<0.001), by 400% at 1-Post (p<0.001), and by 197% at 4-Post (p=0.001). Post hoc analyses further indicated that the increase in IL-6 at Post and 1-Post in HYP exceeded the increase in IL-6 at these same timepoints in NORM ($p<0.001$ and $p=0.006$, respectively).

Interleukin 10 (IL-10). The interaction between study condition and exercise time was significant for IL-10 [$F(3,27) = 4.652$, p=0.010, $\eta_p^2 = 0.341$] (*Fig 4D*). Exercise in NORM did not increase IL-10 until 1-Post exercise (+53%; p=0.023). Whereas, in HYP IL-10 increased by 127% at Post (p=0.001) and by 128% at 1-Post (p=0.001). Post hoc analyses further indicated that the increase in IL-10 at Post and 1-Post in HYP exceeded IL-10 levels at these same timepoints in NORM ($p=0.003$ and $p=0.011$, respectively).

Tumor Necrosis Factor (TNFα). There was a significant main effect of exercise time [$F(3,27) = 7.551$, $p=0.001$, $\eta_p^2 = 0.456$] for TNFα but the main effect of study condition [$F(1,9) = 0.410$, $p=0.538$] and the interaction effect [$F(3,27) = 0.550$, $p=0.653$] were not significant (*Fig 4E*). For the main effect of exercise time, post hoc analysis indicated that TNFα increased (p=0.037) from Pre to Post exercise by 3% in NORM and 11% in HYP. Given the small change in TNFα in NORM we also ran each exercise condition independently (as one-way ANOVAs for the main effect of exercise time) to determine if TNFα demonstrated a meaningful rise following exercise in both study conditions. In NORM the main effect of exercise time was not significant [$F(3,27) = 1.575$, $p = 0.218$], whereas in HYP the main effect of exercise time remained significant [$F(3,27) = 5.060$, $p=0.007$, $\eta_p^2 = 0.360$ and post hoc analysis indicated TNFα increased from Pre to Post exercise (p=0.028).

Please Insert Fig 4 Here

DISCUSSION

The present study investigated the cardiopulmonary, metabolic, gastrointestinal, and cytokine responses of recreational runners that were challenged with 1h of moderate-intensity treadmill exercise under NORM ($F_IO_2 = 20.9\%$) and HYP ($F_IO_2 = 13.5\%$) conditions. HYP exercise caused significant desaturation and increased GI barrier permeability as well as circulating cytokine concentrations. These changes would be expected to influence the "open window" that can occur in the hours following endurance exercise training (Kakanis et al. 2010), which remains an area of considerable debate (Campbell and Turner 2018). To our knowledge we are the first to examine

this constellation of variables in response to exercise stress in normobaric hypoxia. For brevity, the discussion section of this manuscript has been subdivided to highlight systems-level physiological responses first, after which information regarding GI barrier changes and circulating cytokine concentrations has been reported.

Systems-Level Physiological Responses.

In the present study SpO_2 averaged 97% at rest and was maintained between 93-95% during NORM exercise. Whereas SpO_2 averaged 85% at rest in HYP and was maintained between 79-81% during exercise. Using an identical normobaric hypoxia chamber (F_IO_2=13.5%), another group has reported average SpO_2 measurements of 97% (outside of chamber), 85% (following 2h passive rest inside chamber), and 80% (immediately following up to 1h of treadmill exercise at a workload equivalent to 70% VO_{2max}) (Caris et al. 2017). Despite this large reduction in SpO_2 during HYP exercise (-16%), participants in the present study exhibited only a modest increase in HR (7bpm; +4%). This finding contrasts with prior work that reported as compared to normoxic exercise, exercise in normobaric hypoxia (F_IO_2=13.5%) causes significant reductions in SpO_2 (81% vs 98%) and marked elevations in HR (149bpm vs 133bpm) (Lee and Thake 2017). The difference in HR response between these two studies is likely due to the present study examining the same participants under normoxic and hypoxic conditions, whereas in the prior work two different experimental groups were examined (Lee and Thake 2017). Large changes in SpO_2 and lesser changes in HR have been reported by another group that examined sprint interval training (six Wingate anaerobic tests (WAnT) interspersed by 4min recovery periods) in normobaric hypoxia (F_IO_2=15.0%) (Richardson et al. 2016). In that study, SpO_2 was maintained at ~97% during sprint interval training in normoxia, whereas it progressively decreased (p<0.05) across six WAnT that were performed in hypoxia (from 86% on WAnT 1 to 77% on WAnT 4) (Richardson et al. 2016). Despite those large differences in SpO_2, no differences in HR were shown between groups (Richardson et al. 2016).

Although hypobaric hypoxia provides a greater physiological stimulus than normobaric hypoxia (Coppel et al. 2015), the V_E response to normobaric hypoxia (both at rest and during exercise) is known to exceed the V_E response to hypobaric hypoxia (Faiss et al. 2013). Therefore, the 17.3 L/min increase (+30%) in V_E that was shown during HYP exercise in the present study is not surprising. This change, which represents the largest physiologic adjustment that occurred during HYP exercise, is similar to the 26% increase in V_E that was recently reported by another group during normobaric hypoxia (F_IO_2=13.7%) exercise at a lower relative intensity (50% VO_{2max}) (Sotiridis et al. 2018). In the present manuscript we also reported significant (p<0.05) increases in R_R (5 bpm; +14%), and V_T (0.17L/min; +10%) during HYP exercise. To our knowledge, these responses to prolonged treadmill exercise in normobaric hypoxia have not been previously characterized. Based on the significant interaction effect for R_R (p<0.001) but not for V_T (p=0.111) and visual differences between these two data sets (for reference, Figure 1), it appears that changes in V_E during HYP exercise are primarily due to elevations in R_R. Given that elevations in VCO_2 were also shown during HYP exercise (0.41 L/min; +18%), hypercapnia likely promotes this mechanism.

Gastrointestinal Barrier Permeability and Circulating Cytokine Concentrations.

I-FABP is a robust biomarker of GI barrier permeability (Morrison et al. 2014) that exhibits strong correlations with splanchnic hypoperfusion (van Wijck et al. 2011), the lactulose/rhamanose gut permeability test (Pugh et al. 2017), and plasma LPS concentrations (Kim et al. 2018). After leaving the gut LPS enters the portal circulation where it activates liver Kupfer cells, which respond by secreting pro-inflammatory cytokines (TNFα, IL-1β, and IL-6) into circulation (Jiang et al. 1999). Monocytes and macrophages, which are strongly responsive to LPS, also make a significant contribution to the TNFα and IL-10 that appears in circulation (Foey et al. 1998). TNFα stimulates pathways that lead to the activation of NF-κB and JNK/AP-1, which in turn coordinate the activities of multiple pro-inflammatory and immunomodulatory genes (Al-Sadi et al. 2016). IL-10 and IL-1Ra suppress inflammation via inhibition of LPS-mediated production of TNFα and IL-1β and through direct inactivation of IL-1β. Oxidative stress promotes the activation of pro- and anti-inflammatory cytokine cascades (Morgan and Liu 2011), and elevated levels of oxidative stress have been shown in conjunction with normobaric hypoxia exposure under resting (Wang et al. 2007) and exercise (Faiss et al. 2013) conditions. As such, it is likely that the combination of GI barrier permeability and oxidative stress were responsible for the elevated cytokine concentrations that were shown following HYP exercise in the present study, where 1h of treadmill exercise at a workload equivalent to 65% normoxic VO_{2max} was shown to confer significant ($p<0.05$) increases in I-FABP (+68%), IL-1Ra (+266%), IL-6 (+473%), IL-10 (+128%), and TNFα (+11%).

These changes are larger than what was reported previously by Lee and Thake (2017) following normobaric hypoxia (F_IO_2=13.5%) exercise, where I-FABP was shown increased by 43%, IL-6 by 324%, and IL-10 by 89% (Lee and Thake 2017). The higher values in the present study are likely due to differences in the exercise mode used (running vs. cycling) as well as the present study's use of a longer exercise duration (60 vs 40min), and higher exercise workload (65% vs 50% of normoxic VO_{2max}). Further corroboration of these changes is provided by another research group that examined changes in plasma cytokine concentrations of 7 physically active men that were challenged with treadmill exercise (50% VO_{2max}) for 1h (Lira et al. 2017). In that study IL-1Ra increased by 77%, IL-6 by 118%, and IL-10 by 117% (Lira et al. 2017). The increase in IL-1Ra that was reported immediately following exercise in that study is less than what was shown at 1-Post HYP exercise in the present work (+252%), suggesting that IL-1Ra levels in that study may have been further elevated if the time-course of serial blood measurements following exercise had been extended.

It is also important to point out that because participants in the present study maintained the same absolute exercise intensity during NORM and HYP exercise, the higher relative intensity during HYP exercise is expected to have influenced the changes in I-FABP and plasma cytokine concentrations that were shown. A well designed study from another research group helps to clarify this statement. In that study, 12 endurance trained males cycled for 75min at a workload corresponding to 70% of their altitude-specific VO_{2max} under normoxic and hypobaric hypoxia (2000m) conditions (Svendsen et al. 2016). From blood samples that were collected before, after, and 2h after exercise in each study condition, it was determined that exercise in hypobaric hypoxia confers an increase

in plasma cortisol levels (which was not increased following normoxic exercise) and a greater decrease in the $CD4^+$:$CD8^+$ T lymphocyte ratio, but does not cause any further elevations in plasma cytokine responses or antigen stimulated cytokine production (Svendsen et al. 2016). Based on those study findings, the authors concluded that a single bout of exercise in hypobaric hypoxia does not pose any additional meaningful threat to immune function over exercising at the same relative intensity in normoxia (Svendsen et al. 2016). While the results of this study are clearly intriguing, the practical relevance of this study design is questionable because the average power output during normobaric hypoxia cycling was 10.5% lower than what participants maintained during normoxic exercise (Svendsen et al. 2016). This did not provide for any meaningful differences in heart rate, blood lactate concentration, or RPE between the normoxic and hypoxic exercise conditions. From a practical perspective, it would be difficult to convince an athlete train in a hypoxic environment at a lower overall exercise intensity, as this strategy would simply trade one stress (exercise) for another (hypoxia).

In addition, when the plasma cytokine concentrations that were provided in Table 1 of that study are converted to relative (i.e. percent) changes, some interesting trends emerge (Svendsen et al. 2016). The reader is cautioned that although the percent change values that are provided below were calculated directly from data provided in Table 1 of the original publication (Svendsen et al. 2016), the original study did not identify any statistical differences between study conditions. Therefore, these data are provided for descriptive purposes only. In that study IL-1Ra fell 17% following normoxic exercise and remained 13% lower than resting values at 2h post exercise. Whereas, IL-1Ra increased 7% following hypoxic exercise and was further increased (+28%) at 2h post exercise (Svendsen et al. 2016). The time-course and directionality of those changes are similar to the present study findings and are also in agreement with prior work in this area (Lira et al. 2017). The second observation is in regard to IL-10, which rose 36% following normoxic exercise but had returned to exactly resting values (i.e. +0%) at 2h post exercise. Whereas, IL-10 values increased by 125% following hypoxic exercise and remained elevated (+59%) at 2h post exercise (Svendsen et al. 2016). The third observation is in regard to TNFα, which had not changed immediately following normoxic exercise (i.e. +0%) but had fallen below baseline (-8%) at 2h post exercise. Whereas, TNFα increased slightly following hypoxic exercise (+4%) and was even higher (+14%) at 2h post exercise (Svendsen et al. 2016). Again, the time-course and directionality of these changes in IL-10 and TNFα following hypoxic exercise are similar to present study findings and also agree with prior work in this area (Lira et al. 2017).

Future Directions.

The normobaric hypoxia exercise protocol that was utilized in the present study caused GI barrier permeability and elevated plasma cytokine levels. In addition to being in agreement with prior work in this area (Lee and Thake 2017; Lira et al. 2017), these findings are also similar to what our group has previously reported following exertional heat stress (Kuennen et al. 2011; McKenna et al. 2017; Szymanski et al. 2018), where hypoxemia in GI tissues (Dokladny et al. 2016) was shown to increase LPS in circulation (Kuennen et al. 2013; Kuennen et al. 2011) and activate the immune system (Kuennen et al. 2013; Kuennen et al. 2011; Lee and Thake 2017;

Szymanski et al. 2018). For context, we recently reported that 1h of treadmill exercise (65% VO_{2max}) in hot (37°C), dry (25% relative humidity) conditions causes significant (p<0.05) increases in I-FABP (+87%), IL-1Ra (+153%), IL-6 (+93%), IL-10 (+59%), and TNFα (+24%) (Szymanski et al. 2018). Given that GI barrier permeability is a known contributor to the pathogenesis of exertional heat stroke (Lim and Mackinnon 2006), it is plausible that a similar mechanism may confer increased risk for acute mountain sickness symptoms during exercise at simulated altitude. Elite athletes could be at even greater risk for this issue, as their enhanced muscular oxygen extraction capabilities have been shown to confer exaggerated hypoxemic responses during normobaric hypoxia exercise (Van Thienen and Hespel 2016). For that reason, future work should add to the present study findings by examining soluble and cellular markers of innate and adaptive immune responses, which would help to clarify the net effect GI barrier permeability has on a runner's immune status (Campbell and Turner 2018). In the event that immunosuppression is shown, it would also be worthwhile to determine if some of the nutritional countermeasures that influence GI barrier permeability and associated immune responses during exertional heat stress (Kuennen et al. 2011; Kuennen et al. 2015; McKenna et al. 2017; Szymanski et al. 2018) exert similar effects during exercise at simulated altitude. It is common for recreational athletes to utilize one or more dietary supplements in conjunction with their exercise plan (Borrione et al. 2012; Granados et al. 2014), suggesting that the appropriateness of ingesting certain dietary supplements (Granados et al. 2014; Kuennen et al. 2011; McKenna et al. 2017) in conjunction with normobaric hypoxia exercise may also warrant further attention.

Limitations.

Participants exercised in a fasted state because foodstuff ingestion influences gastrointestinal barrier function (Sanford and Smyth 1974). Although necessary, this control may reduce the generalizability of study findings to real-world athlete scenarios. Future research should examine the impact of food consumption in general as well as specific nutrients on the HYP exercise outcomes that were examined in the present manuscript. The source of IL-6 in circulation is unknown. Therefore, elevations in circulating IL-6 following HYP exercise could be a byproduct of greater LPS in circulation, as this has been shown previously (Selkirk et al. 2008). Alternatively, given the higher RER that was shown during HYP exercise in the present study, as well as prior work reporting increased energy expenditure during work at high altitude (Hill et al. 2011) and IL-6's known role as an extracellular energy sensor (Pedersen 2012), this might also reflect HYP promoting greater IL-6 release from skeletal muscle on account of the greater cellular energy demand.

Short Summary of Significance of Work and Conclusions Drawn.

Submaximal treadmill exercise (65%VO_{2max}) in normobaric hypoxia (F_IO_2=13.5%) increases the cardiopulmonary responses of recreational runners by 10-30% over matched workload exercise performed in normoxia (F_IO_2=20.9%). Although significant desaturation was noted, all participants were able to complete the full 1h of treadmill exercise and qualitative data (RPE) indicate this exercise challenge was well tolerated. Gut barrier permeability (I-FABP) and circulating concentrations of pro-inflammatory (IL-6 and TNFα) and anti-inflammatory (IL-1Ra, IL-10) cytokines were also shown to be elevated, leaving the "window open" with regard

to the net effect these changes have on host immune parameters during the exercise recovery period. Future quantification of circulating markers of leukocyte activation and identification of adjustments in leukocyte protein expression during the exercise recovery period will go a long way towards answering this important research question.

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CONLICT OF INTEREST

The authors have no conflicts of interest to report.

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Table 1. Sensitivity and Precision of Enzyme Linked Immunosorbant Assays.

Blood Marker	Product Number	Detection Limit	Intra-Assay CV	Inter-Assay CV
I-FABP	HK406-02	47pg/ml	5.6%	8.0%
IL-1Ra	DRA00B	6.3pg/ml	4.0%	8.5%
IL-6	HS600C	0.03pg/ml	3.0%	8.4%
IL-10	HS100C	0.09pg/ml	3.7%	9.3%
TNFα	HSTA00E	0.02pg/ml	5.2%	9.5%

Table 2. Equality of Study Conditions

Category	Variable	Normoxia	Hypoxia	T-Test
Nutrition	Total energy intake (mJ/d)	8.6 ± 0.4	8.7 ± 0.5	0.434
	Carbohydrate (g/d)	229 ± 20	240 ± 20	0.410
	Fat (g/d)	80 ± 7	80 ± 8	0.990
	Protein (g/d)	102 ± 7	101 ± 7	0.722
Ambient	Temperature (°C)	21.4 ± 0.2	21.5 ± 0.2	0.669
	Relative Humidity (%)	33.0 ± 2.3	36.6 ± 2.7	0.076
Exercise	Running Speed (km/h)	9.3 ± 0.3	9.3 ± 0.3	0.929
	VO_{2max} (%)	63.9 ± 2.0	64.5 ± 2.1	0.838
Urine	Urine Specific Gravity	1.020 ± 0.002	1.020 ± 0.002	0.918
Blood	Pre Hematocrit (%)	47.6 ± 0.6	48.0 ± 0.9	0.706
	Post Hematocrit (%)	47.1 ± 0.6	47.4 ± 0.9	0.778
	Pre Hemoglobin (g/dl)	15.9 ± 0.2	16.0 ± 0.3	0.711
	Post Hemoglobin (g/dl)	15.7 ± 0.2	15.8 ± 0.3	0.761
	Δ Plasma Volume (%)	1.0 ± 0.9	1.3 ± 1.1	0.839

All data are mean ± SE for N=10.

Table 3. Absolute and Relative Changes in Systems-Level Physiological Responses

Variable	Normoxia	Hypoxia	Difference	P Value
Minute Ventilation (L/min)	58.6 ± 3.2	75.9 ± 4.8	+30%	<0.001
Respiratory Rate (bpm)	37 ± 3	42 ± 4	+14%	<0.001
Tidal Volume (L/min)	1.79 ± 0.13	1.96 ± 0.13	+10%	0.023
O_2 Consumption (L/min)	2.73 ± 0.17	2.79 ± 0.20	+2%	0.155
CO_2 Production (L/min)	2.33 ± 0.14	2.74 ± 0.19	+18%	<0.001
Respiratory Exchange Ratio	0.86 ± 0.01	0.99 ± 0.03	+15%	<0.001
Energy Expenditure (kcal/min)	13.5 ± 0.8	14.0 ± 1.0	+4%	0.053
Carbohydrate Oxidation (g/min)	1.73 ± 0.13	3.29 ± 0.28	+90%	<0.001
Lipid Oxidation (g/min)	0.66 ± 0.07	0.17 ± 0.05	-74%	<0.001
Heart Rate (bpm)	166 ± 4	173 ± 3	+4%	0.003
Peripheral O_2 Saturation (%)	95 ± 1	80 ± 1	-16%	<0.001
Absolute Tissue Saturation (%)	68 ± 2	61 ± 3	-10%	0.027
Rating of Perceived Exertion	12 ± 1	14 ± 1	+17%	<0.001

Mean responses over 60min of treadmill exercise in Normoxia (F_IO_2=20.9%) and Hypoxia (F_IO_2=13.5%) for N = 10 study participants. Data are reported as mean ± SE.

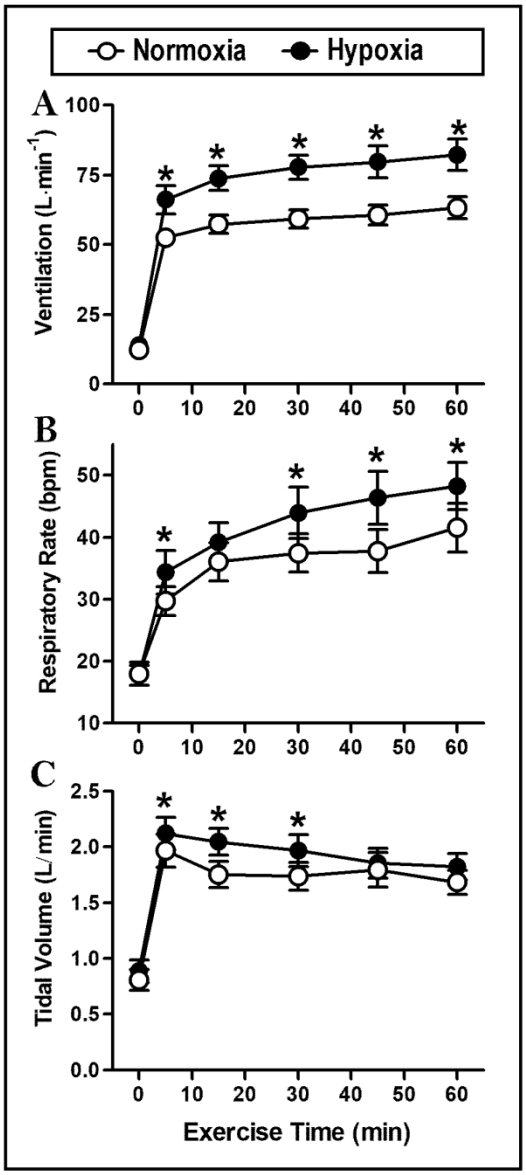
FIGURE CAPTIONS.

Figure 1. Ventilatory responses to one hour of treadmill exercise in normoxia (F_IO_2 = 20.9%) and normobaric hypoxia (F_IO_2 = 13.5%). (A) Minute ventilation; (B) respiratory rate; and (C) tidal volume during 60 min of treadmill exercise performed at a workload equivalent to 65% $VO_{2max.}$ Data are mean ± SEM for N = 10. Data were analyzed with two-factor RM-ANOVAs, where intervention (Normoxia or Hypoxia) and exercise time (0-60 min) served as the repeated measures factors. Statistical significance was set at $p \leq 0.05$. Significant main and interaction effects were further evaluated using Newman Keuls post hocs, as described in Methods. *indicates $p \leq 0.05$ compared with Normoxia.

Figure 2. Metabolic responses to one hour of treadmill exercise in normoxia (F_IO_2 = 20.9%) and normobaric hypoxia (F_IO_2 = 13.5%). (A) Volume of oxygen consumption (VO_2); (B) volume of carbon dioxide production (VCO_2); (C) respiratory quotient; (D) energy cost; (E) carbohydrate (CHO) oxidation; and (F) fat oxidation during 60 min of treadmill exercise performed at a workload equivalent to 65% VO_{2max}. Data are mean ± SEM for N = 10. Data were analyzed with two-factor RM-ANOVAs, where intervention (Normoxia or Hypoxia) and exercise time (0-60 min) served as the repeated measures factors. Statistical significance was set at $p \leq 0.05$. Significant main and interaction effects were further evaluated using Newman Keuls post hocs, as described in Methods. *indicates $p \leq 0.05$ compared with Normoxia.

Figure 3. Cardiovascular responses and perceived effort during one hour of treadmill exercise in normoxia (F_IO_2 = 20.9%) and normobaric hypoxia (F_IO_2 = 13.5%). (A) Peripheral oxygen saturation (SpO_2); (B) absolute tissue saturation (StO_2); (C) heart rate; (D) rating of perceived exertion (Borg scale) during 60 min of treadmill exercise performed at a workload equivalent to 65% VO_{2max}. Data are mean ± SEM for N = 10. Data were analyzed with two-factor RM-ANOVAs, where intervention (Normoxia or Hypoxia) and exercise time (0-60 min) served as the repeated measures factors. Statistical significance was set at $p \leq 0.05$. Significant main and interaction effects were further evaluated using Newman Keuls post hocs, as described in Methods. *indicates $p \leq 0.05$ compared with Normoxia.

Figure 4. Gastrointestinal barrier damage and circulating cytokine responses before, during, and after one hour of treadmill exercise in normoxia (F_IO_2 = 20.9%) and normobaric hypoxia (F_IO_2 = 13.5%). (A) Intestinal fatty acid binding protein (I-FABP); (B) Interleukin 1 receptor antagonist (IL-1Ra); (C) Interleukin 6 (IL-6); (D) Interleukin 10 (IL-10); and (E) Tumor necrosis factor α (TNFα) concentration in blood samples collected before (Pre), after (Post), 1-hour after (1-Post), and 4-hours after (4-post) 60 min of treadmill exercise performed at a workload equivalent to 65% $VO_{2max.}$ Data for subjects (N = 10) are presented as box plots that display individual data points (open circles), the 25 and 75th interquartile ranges (boxes), and the median (mid-line). Whiskers illustrate the highest and lowest value. Data were analyzed with two-factor RM-ANOVAs, where intervention (Normoxia or Hypoxia) and blood sample timepoint (Pre, Post, 1Post, and 4Post) served as the repeated measures factors. Main effects and interaction effects are reported above the respective data sets. Statistical significance was set at $p \leq 0.05$. Significant effects were further evaluated using Newman Keuls post hocs. * indicates $p \leq 0.05$ compared with Pre in the same study condition. $^{\neq}$indicates $p \leq 0.05$ compared with the same timepoint in the opposite study condition.



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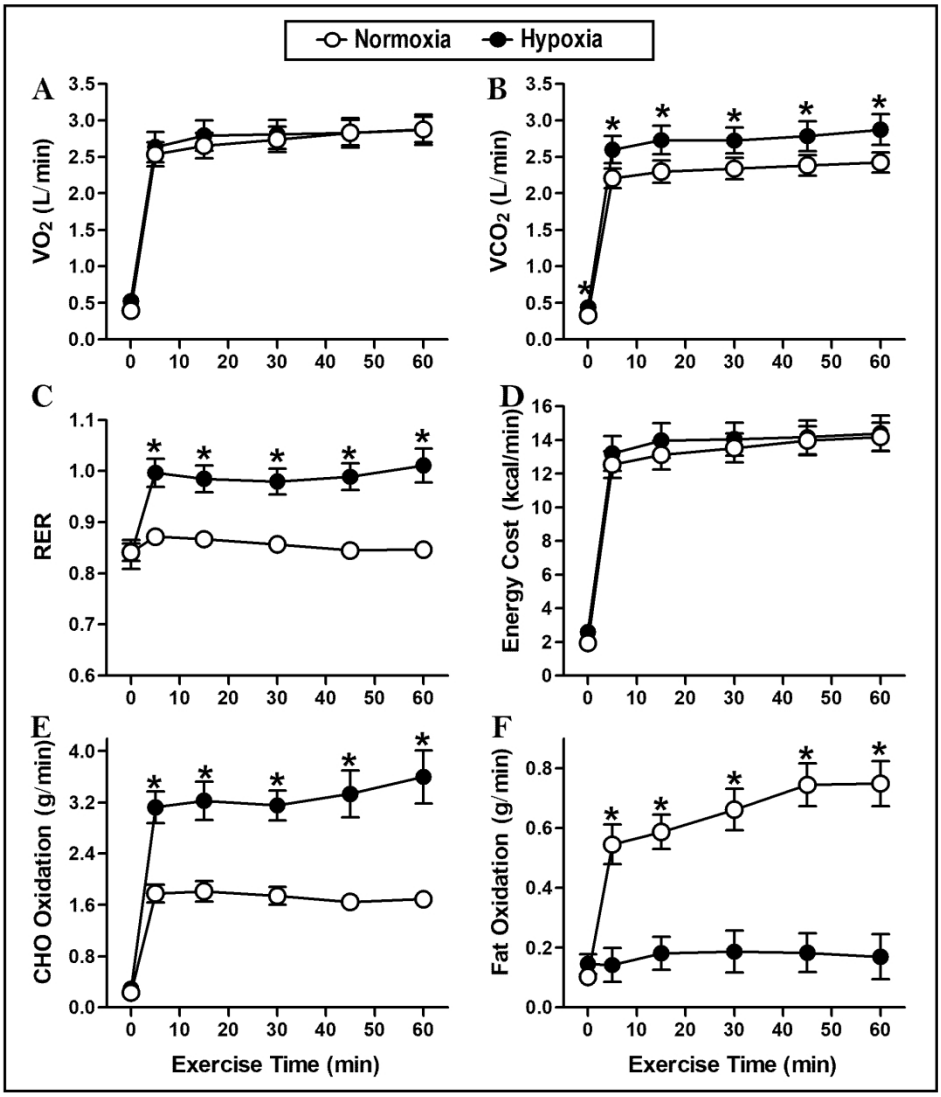

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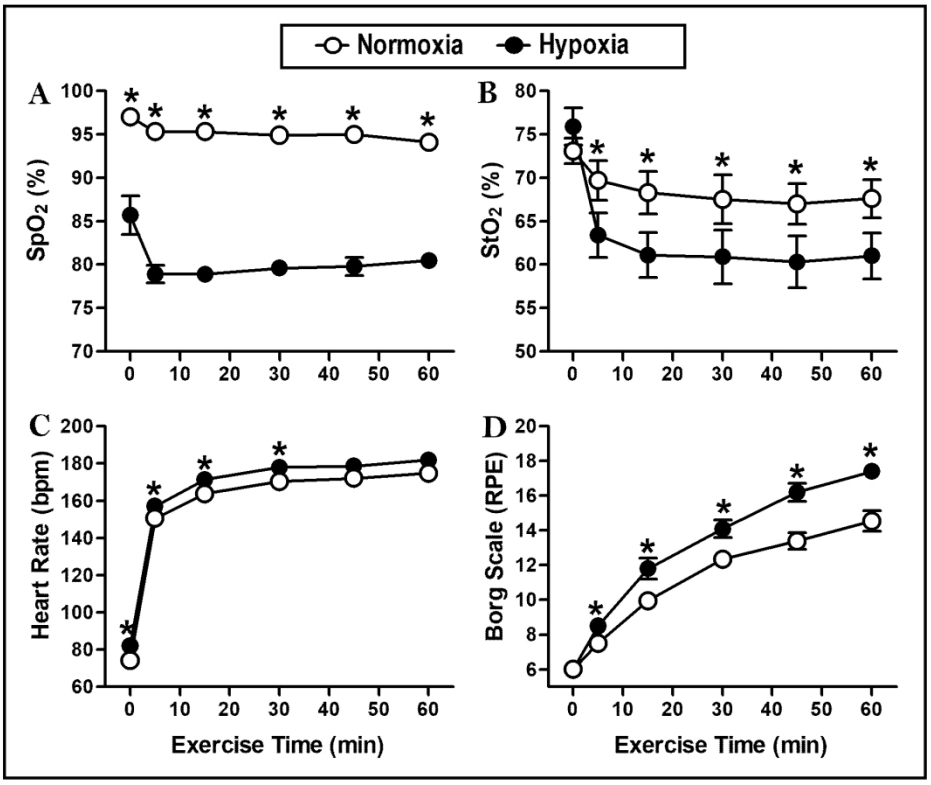

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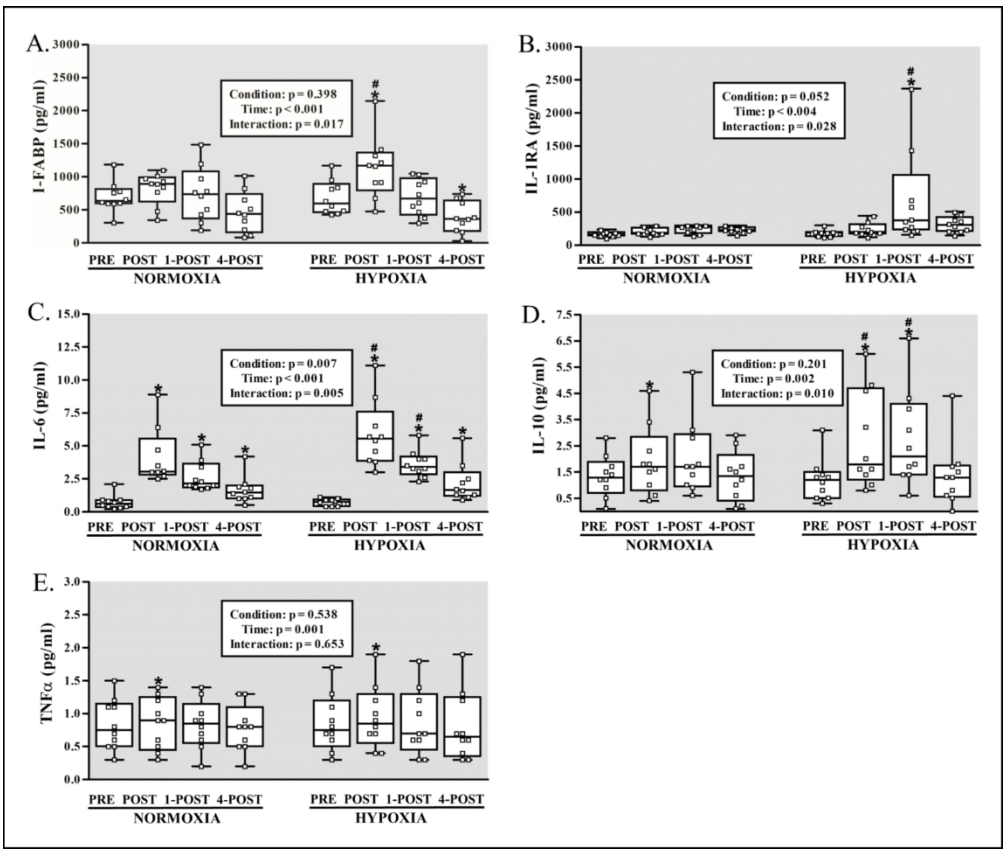

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